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Chapter 7

BACTERIAL GROWTH ON HALOGENATED ALIPHATIC HYDROCARBONS: GENETICS AND BIOCHEMISTRY

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1. INTRODUCTION

Many synthetically produced halogenated aliphatic compounds are xenobiotic chemicals in the sense that they do not naturally occur on earth at biologically significant concentrations. Nevertheless, various microorganisms have been isolated that possess the capacity to grow at the expense of these compounds, and to use them as a carbon and energy source under aerobic conditions. This raises a number of interesting questions concerning the mechanisms of dehalogenation, and the evolution and distribution of dehalogenase-encoding genes. For example, to what extent were the biochemical pathways for the mineralization of such xenobiotic halogenated chemicals preexisting before their large-scale anthropogenic introduction into the environment? What adaptation events at the genetic level occurred after the release of the xenobiotics? What are the origins of enzymes that cleave carbon-halogen bonds? Did similar mechanisms evolve in different organisms, at different sites, or were genes and organisms distributed from one site to another?

Over the last few decades, several classes of reactions for the dehalogenation of xenobiotic compounds have been found, and in some cases the enzymatic mechanisms have been unraveled to the atomic level by x-ray crystallographic studies. At the same time, genetic studies have revealed much about the diversity, similarities, and possible distribution mechanisms of dehalogenase genes. This review focuses on the catabolic pathways for the aerobic mineralization of halogenated aliphatics, including dehalogenation reactions and issues of adaptation and distribution, with an emphasis on events that may have occurred after introduction of these xenobiotics into the environment.

Bacterial growth on halogenated compounds requires the presence of enzymes that are capable of cleaving carbon-halogen bonds. The mechanisms and diversity of some

of these enzymes have been studied in detail. Furthermore, genetic analysis has yielded insight into the evolutionary relationships of dehalogenase genes and their distribution. This allows one to understand genetic events that have contributed to the recruitment of catabolic pathways for synthetic compounds. In this chapter, the most important principles are illustrated with enzymes and genes involved in the degradation of xenobiotic organohalogenes such as dichloromethane, 1,2-dichloroethane, 1,2-dibromoethane, and 1,3-dichloropropylene.

2. AEROBIC DEGRADATION OF HALOALIPHATICS

Most halogenated aliphatic compounds that occur as environmental pollutants should, from a thermodynamic point of view, be suitable growth substrates for aerobic bacteria (7). Indeed, a large number of aerobic bacterial cultures that utilize haloaliphatics with a low degree of halogenation (1-3 halogens per molecule) have been found. Their generation times may be in the range of 2 to 10 hours, which makes their cultivation for physiological studies convenient. Even some highly chlorinated compounds can serve as a growth substrate, e.g., dichloromethane, trichloroacetate, and hexachlorocyclohexane (Figure 7.1).

Enrichment trials and degradation studies have also shown that such bacterial cultures can only be obtained for a restricted number of haloaliphatics, whereas many other chlorinated compounds appear to be refractory to degradation. Recalcitrance may either be due to the lack of an enzyme that can catalyze the first step, often a dehalogenation reaction, or to the lack of a productive metabolic pathway. If dehalogenation is not the first step, there is a high risk of formation of toxic intermediates which may prohibit the use of a compound as growth substrate. Examples include the difficulties of obtaining growth on substrates such as 1,2-dibromoethane and chloroethenes. Modified pathways following different metabolic strategies may be used to prevent the formation of toxic intermediates. For example, the conversion of 1,2-dichloroethane and 1,2-dibromoethane starts with a hydrolytic cleavage by a haloalkane dehalogenase for both compounds, resulting in their corresponding alcohols. Whereas 1,2-dichloroethane-degrading *Xanthobacter* strains further oxidize the 2-chloroethanol product by an alcohol dehydrogenase, the 2-bromoethanol formed from 1,2-dibromoethane in a 1,2-dibromoethane-utilizing *Mycobacterium* is directly dehalogenated by a halohydrin lyase to epoxyethane. In this way, the accumulation of the highly toxic intermediate bromoacetaldehyde is prevented (42).

Some of the most important pathways for the aerobic degradation of haloaliphatics, and the role of dehalogenases are presented in Figure 7.1. It appears that the actual dehalogenation reactions are very diverse. Dehalogenation may proceed, for example, by substitution (e.g., with water), elimination of HCl, or by reduced cofactor-dependent oxidative conversion. Hydrolytic dehalogenases have received considerable attention, and x-ray structures of some of these enzymes have been determined from which detailed mechanistic insight has been obtained.

Table 7.1. Classification of aliphatic dehalogenases.

Structure/protein family	Mechanism	Examples, accession numbers	Substrate	References
Haloalkane dehalogenases. α/β -hydrolase fold super family	Hydrolysis with formation of an ester intermediate.	Haloalkane dehalogenase Dh1A	1,2-Dichloroethane	20, 63
	Covalent catalysis involving a catalytic triad with Asp as the nucleophile	<i>Xanthobacter autotrophicus</i> GJ10 (M26950)		
		Haloalkane dehalogenase DhaA	1-Chlorobutane, 1,2-dibromoethane, 1,3-dichloropropene	26, 38
		<i>Rhodococcus erythropolis</i> NCIMB 13064 (AF060871)		
HAD (haloacid dehalogenase) super family. Group II dehalogenases (similar to phosphatase domain).		1,3,4,6-Tetrachloro-1,4-cyclohexadiene halidehydrolase LinB	1,3,4,6-Tetrachloro-1,4-cyclohexadiene, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol	31, 32
		<i>Sphingomonas paucimobilis</i> UT26 (D14594)		
	Hydrolysis with inversion of product configuration.	Haloacid dehalogenase Dh1B	L-2-Chloropropionic acid, 2-chloroacetate	46
	Covalent catalysis involving a nucleophilic Asp	<i>Xanthobacter autotrophicus</i> GJ10 (M81691, X84038)		
		Haloacid dehalogenase L-DEX	L-2-Chloropropionic acid, 2-chloroacetate	16, 29
		<i>Pseudomonas</i> sp. YL (S74078)		
		Haloacetate dehalogenase DehH2	2-chloroacetate	23
		<i>Moraxella</i> sp. B (D90423)		

DL-DEX type (Group I dehalogenases)	Hydrolysis with inversion of product configuration without formation of an ester intermediate. Non-covalent catalysis	Haloacid dehalogenase (DL-DEX) <i>Pseudomonas</i> sp. 113 (U97030)	L-2-Chloropropionic acid, 36, 37 D-2-chloropropionic acid, 2-chloroacetate
		Haloacid dehalogenase (HadD) <i>Pseudomonas putida</i> AJ1 (M81841)	D-2-Chloropropionic acid, 2 2-chloroacetate
Halohydrin lyase (similar to short-chain reductase/dehydrogenase) family).	Intramolecular substitution yielding epoxides	Halohydrin lyase (haloalcohol dehalogenase, halohydrin epoxidase) (HheA) <i>Corynebacterium</i> sp. N1074 (D90349)	2,3-Dichloro-1-propanol, 1,3-dichloro-2-propanol, 3-chloro-1,2-propanediol
		Halohydrin lyase (haloalcohol dehalogenase, halohydrin epoxidase) (HheB) <i>Corynebacterium</i> sp. N1074 (D90350)	
Dichloromethane dehalogenase (similar to glutathione S-transferase family, θ -class)	Nucleophilic substitution by glutathione	Dichloromethane dehalogenase (DcmA) <i>Methylobacterium</i> sp. DM4 (M32346) Type I Dichloromethane dehalogenase (DcmA) <i>Methylophilus</i> sp. DM11 (L26544) Type II	Dichloromethane 1, 27

Table 7.1. (continued)

Structure/protein family	Mechanism	Examples, accession numbers	Substrate	References
Chloroacrylic acid dehalogenases	Hydration of the C=C bond, yielding a possible chlorohydrin intermediate from which HCl is eliminated to give malonate semialdehyde	<i>cis</i> -3-Chloroacrylic acid dehalogenase <i>Burkholderia (Pseudomonas) cepacia</i> CAA, <i>Corynebacterium</i> sp. No sequence information available	<i>cis</i> -3-Chloroacrylic acid	13, 60
		<i>trans</i> -3-Chloroacrylic acid dehalogenase <i>Corynebacterium</i> sp. FG41 No sequence information available	<i>trans</i> -3-Chloroacrylic acid	
Hexachlorocyclohexane dehydrochlorinase	Elimination of HCl, leading to formation of a double bond (dehydrohalogenation)	Hexachlorocyclohexane dehydrochlorinase (LinA) <i>Sphingomonas paucimobilis</i> UT26 (D90355)	γ -Hexachlorocyclohexane	32, 33
Methyltransferase	Methyl transfer to acceptor ion (HS ⁻)	<i>Methylobacterium</i> sp. CC495	Chloromethane	4
Methyltransferase	Transfer to tetrahydrofolate	<i>Methylobacterium</i> sp. CM4 (AJ011317)	Chloromethane	53, 61
Monooxygenase	Oxygenolytic dehalogenation	Methyl chloride monooxygenase. <i>Hyphomicrobium</i> sp. MC1 No sequence information available	Chloromethane	14
		Chloroalkane monooxygenase (Mox) <i>Pseudomonas</i> sp. DCA1 No sequence information available	1,2-Dichloroethane	10

3. CLASSIFICATION AND MECHANISTIC ASPECTS OF DEHALOGENASES

Traditionally, dehalogenases have been classified according to their substrate range. Such a classification scheme places haloacid dehalogenases, haloacetate dehalogenases, and haloalkane dehalogenases in different groups. We think this classification should be abandoned, since more recent information obtained from sequence analysis has shown that dehalogenases can be ordered into different homology groups, corresponding to the structural folds and catalytic mechanisms of the enzymes, rather than their observed substrate range. For example, fluoroacetate dehalogenase from a *Moraxella* strain (DehH1) (23) appears to be structurally more similar to several haloalkane dehalogenases which are also α/β -hydrolase fold enzymes, than to most haloacid dehalogenases or haloacetate dehalogenases. Furthermore, enzymes that convert chloroacetate can be divided in different groups, some using a covalent mechanism whereas others not forming a covalent intermediate. In this case, a classification scheme according to substrate range provides little information on the evolution and distribution of enzymes and metabolic pathways. An overview of dehalogenase types, their relationship to other enzymes of the same class, and their genes and accession numbers, is given in Table 7.1.

The first well-characterized dehalogenase, with respect to structure and mechanism, was that of *Xanthobacter autotrophicus* GJ10, an organism isolated in 1983 in The Netherlands (19). The enzyme, Dh1A, catalyzes dehalogenation of a variety of short chain haloalkanes. The reaction mechanism of Dh1A is characterized by covalent catalysis, with an Asp-His-Asp catalytic triad similar to that found in the classical chymotrypsin-like serine proteases as the most salient feature (Figure 7.2). In the first step, there is a nucleophilic displacement in which the carbon atom of the substrate to which the halogen atom is bound is attacked by the nucleophile aspartate residue (Asp), thereby forming a covalent alkyl-enzyme ester intermediate (63). In the second step, the histidine base residue (His), assisted by the second Asp which acts as a charge-relay residue, activates a water molecule that hydrolyzes the ester intermediate by attacking the carbonyl carbon atom of the enzyme. The most important difference between this mechanism and that of serine proteases is that the first and second nucleophilic substitutions occur on different carbon atoms in the dehalogenase enzyme, whereas in proteases they occur on the same carbon. In agreement with this, the carbonyl function required for hydrolysis of the covalent intermediate, is supplied by the dehalogenase enzyme, rather than by the substrate as in serine hydrolases (44). In addition to the catalytic triad, the haloalkane dehalogenase possesses two tryptophan (Trp) residues that are involved in leaving group stabilization. Thus, the enzyme is really evolved to carry out a dehalogenation reaction, and it is not a general hydrolase that fortuitously also dechlorinates.

Other haloalkane dehalogenases have been found in various *Rhodococcus* strains (26, 41). The DhaA enzyme of strain NCIMB13064 has a different substrate range than Dh1A. The former enzyme also converts long-chain chloroalkanes, but its activity with 1,2-dichloroethane is very low. Both Dh1A and DhaA enzymes belong to the α/β -hydrolase fold superfamily, a group to which also epoxide hydrolases belong. Other enzymes belonging to this group include DehH1 from *Moraxella* sp. strain B, a haloacetate dehalogenase which converts both fluoro- and chloroacetate, and LinB, a

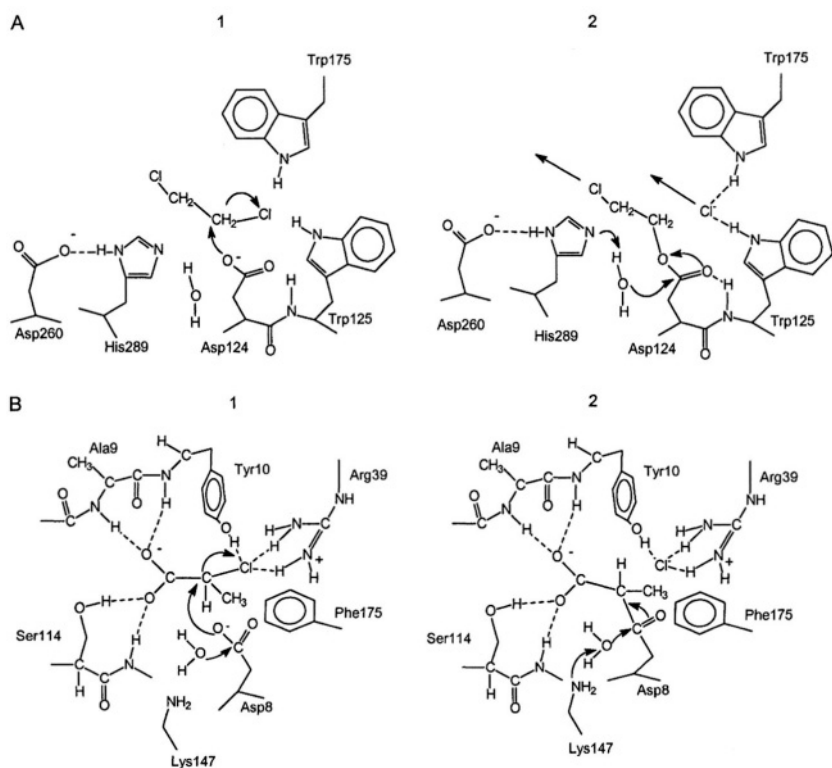


Figure 7.2. Catalytic mechanisms of *Xanthobacter autotrophicus* GJ10 haloalkane dehalogenase (DhlA, top) and haloacid dehalogenase (DhlB, bottom). In both cases, a conserved aspartic acid residue is responsible for formation of the covalent intermediate. Different groups are involved in halide stabilization (44, 46, 63).

halohydratase involved in the degradation of γ -hexachlorocyclohexane in *Sphingomonas paucimobilis* (Figure 7.1). Group classification is based on sequence similarity and use of a similar hydrolytic mechanism with involvement of a catalytic triad (17, 30, 31). In DhaA and LinB, the catalytic triad consists of Asp-His-Glu, with the glutamate residue (Glu) acting as the charge-relay acid rather than an Asp as in DhlA of *X. autotrophicus* (17, 38). In these enzymes, the catalytic Glu is located after β strand 6, which is a different position than that of the catalytic Asp of DhlA, which is located behind β strand 7 (6). It has been shown that the position of this charge relay residue can be moved in DhlA from behind β strand 7 to a position behind β strand 6 (24).

Haloacid dehalogenases (HAD) are classified into at least two groups, which are evolutionarily unrelated (15). The HAD-type haloacid dehalogenases (group II) are the best characterized enzymes. These have a three-dimensional structure which is different from that found in haloalkane dehalogenases, but hydrolysis also proceeds via covalent catalysis (Figure 7.2). This class of dehalogenases has a topological fold which is similar

to the one found in several phosphatases, and the conserved nucleophilic Asp residue involved in the displacement of the halogen is located close to the *N*-terminus. DhlB of *X. autotrophicus* GJ10 and L-DEX of *Pseudomonas* sp. YL are examples where x-ray structures have been determined (16, 29, 46). These group II enzymes usually convert *L*-2-chloropropionic acid, but not *D*-2-chloropropionic acid, always with inversion of product configuration. Haloacetate dehalogenase DehH2 from *Moraxella* sp. B also belongs to this group, although substrate usage by this enzyme is non-stereospecific, illustrating that stereoselectivity and substrate range are not well correlated with structural and mechanistic features (23).

Besides covalent catalysis, non-covalent catalysis has also been proposed for haloacid dehalogenases. There are indications that with the dehalogenase DL-DEX from *Pseudomonas* sp. strain 113, hydrolysis does not proceed via formation of a covalent ester intermediate, but the hydroxyl group of water directly attacks the α -carbon of 2-haloalkanoic acid to displace the halogen atom (37). This protein belongs to a group of homologous haloacid dehalogenases (group I) that includes enzymes which convert both *L*-2-chloropropionic acid and *D*-2-chloropropionic acid, as well as enzymes which convert only *D*-2-chloropropionic acid with inversion of product configuration (2, 15). The group also includes a dehalogenase enzyme involved in the conversion of trihaloacetate to carbon monoxide (52). Other hydrolytic dehalogenases with activity towards chloroacetate and chloropropionates probably exist, but sequence data and structural information are too scarce to allow adequate classification (15).

Halohydrin lyases (also called haloalcohol dehalogenases) provide another dehalogenation mechanism that uses intramolecular substitution, as is found in *Corynebacterium* sp. N-1074. These proteins share partial sequence similarity with proteins of the short-chain reductase/dehydrogenase (SDR) family. Such an enzyme is involved in the degradation of 1,3-dichloro-2-propanol, where two halohydrin-lyase mediated dehalogenation steps yield an intermediary epoxide (Figure 7.1) (34, 35). Different halohydrin lyases in *Corynebacterium* sp. N-1074 have been described, which display different enantioselectivities. For example, HheA acts non-stereospecifically, while HheB is enantioselective, converting mostly the (*R*)-enantiomer from a racemic substrate mixture. The HheA and HheB enzymes show no significant structural similarity, except in the carboxyl-terminal region (65). Possibly, they share similar catalytic mechanisms but have major differences in substrate recognition domains.

Dehalogenation by dichloromethane dehalogenase (DcmA) of *Methylobacterium* sp. DM4 and *Methylophilus* sp. DM11 involves a thiolytic substitution reaction (Figure 7.1). DcmA is an inducible enzyme enabling bacterial growth on dichloromethane as the sole carbon and energy source. All known dichloromethane dehalogenases belong to the glutathione S-transferase super family (***θ* class**). The enzymatic mechanism involves a nucleophilic displacement of halogen (Cl) by glutathione, rather than by a hydroxyl or carboxylate oxygen, producing a halomethylthioether intermediate which is converted to formaldehyde (3). Two DcmA gene types have been found which are not directly related to each other. Comparison of strain DM11 and strain DM4 dichloromethane dehalogenase sequences revealed 56% identity at the protein level, thus indicating an ancient divergence of the two enzymes (1). Also structural differences between the two enzymes exist. The *Methylobacterium* sp. DM4 enzyme has a subunit molecular mass of 33 kDa and an α_6 structure, while the *Methylophilus* sp. DM11 enzyme has a subunit

mass of 34 kDa and an α_2 structure (25, 27).

Chloromethane has been shown to be degraded and utilized via formate by the facultative methylotrophic bacterium CC495 under aerobic conditions (4). The dehalogenating enzyme appeared to be an inducible 67 kDa methyltransferase with a corrinoid-bound cobalt atom. In the reaction, the methyl group of chloromethane appears to be transferred to a sulfhydryl acceptor (HS^+), yielding methanethiol, which can be converted to formaldehyde by a methanethiol oxidase (Figure 7.1G). The methyltransferase enzyme shows mechanistic similarities to cobalamin-dependent methionine synthase from *Escherichia coli*. Alternatively, in *Methylobacterium* sp. the methyltransferase becomes methylated and subsequently donates the methyl group to tetrahydrofolate in a reaction catalyzed by another methyltransferase (61). This second methyltransferase was recently purified and accepted methylcobalamin as the methyl donor (53). Further metabolism then proceeds via a pterine-dependent pathway.

Chloroacrylic acid dehalogenase of *Pseudomonas pavonaceae* is the second dehalogenating enzyme in the degradation pathway of 1,3-dichloropropene (43) (Figure 7.1C). A formal hydration reaction at the C=C bond of 3-chloroacrylic acid takes place, yielding the unstable intermediate 3-chloro-3-hydroxypropanoic acid, from which HCl is eliminated to give malonate semialdehyde (13). In a coryneform strain, two different enzymes are present, which convert either *cis*- or *trans*-3-chloroacrylic acid (60). The *cis*-3-chloroacrylic acid dehalogenase is a 38 kDa trimeric protein, while the *trans*-3-chloroacrylic acid dehalogenase consists of 7.4 and 8.7 kDa subunits.

In the first two steps in the γ -hexachlorocyclohexane degradation pathway of *Sphingomonas paucimobilis* (Figure 7.1E), a dehydrohalogenation reaction catalysed by hexachlorocyclohexane dehydrochlorinase (LinA) eliminates two biaxial HCl pairs in two sequential steps, leading to the formation of double bonds in the substrate (33, 55). LinA is a homotetramer consisting of 16.5 kDa subunits. The *linA* gene shows no homology to other known dehalogenase genes (18). However, the involvement in catalysis of a histidine residue (His73) as a base that abstracts a proton from the substrate, facilitated by Asp25, was proposed on the basis of similarity to a hydratase and isomerase with known x-ray structures (55). How the enzyme interacts with the halogen is at present unknown. The product formed by LinA, 1,3,4,6-tetrachloro-1,4-cyclohexadiene, is converted by the halidohydrolase LinB, an enzyme of the α/β -hydrolase fold superfamily, as discussed above.

Vinyl chloride, a compound that is produced on a large scale by industry and is also biologically formed during the reductive breakdown of chlorinated ethenes, can be degraded oxidatively. Many organisms have been described that degrade vinyl chloride cometabolically during utilization of other compounds, but so far only two strains, *Mycobacterium aurum* L1 and *Pseudomonas aeruginosa* MF1, have been isolated which grow on vinyl chloride as the sole carbon and energy source (12, 62). Vinyl chloride breakdown in *M. aurum* L1 was shown to be mediated by an alkene monooxygenase, yielding chlorooxirane, which is presumably further degraded by an epoxide dehydrogenase (11). In this pathway, the monooxygenase-mediated conversion is not the dehalogenating reaction.

In the oxygenolytic degradation of chloromethane and 1,2-dichloroethane, the first degradation step is most likely catalyzed by a dehalogenating monooxygenase. Chloromethane serves as a sole carbon and energy source for growth of

Hyphomicrobium MC1 (14). Degradation is possibly catalyzed by methyl chloride monooxygenase, yielding formaldehyde. Recently, a *Pseudomonas* strain (DCA1) was described that grows aerobically on 1,2-dichloroethane as the sole carbon source (Figure 7.1). The first step of the 1,2-dichloroethane degradation pathway is thought to be mediated by a monooxygenase because the reaction is dependent of oxygen and NADH (10). In the proposed pathway, oxidation of 1,2-dichloroethane yields chloroacetaldehyde, which is most likely further degraded to glycolate. This is analogous to the 1,2-dichloroethane degradation pathway described in *X. autotrophicus* GJ10. At the moment, no DNA sequences or information on protein structure or reaction mechanisms of the monooxygenase proteins involved in the dehalogenation reactions are available.

4. RECENT EVOLUTION OF HALOALKANE DEHALOGENASE

The evolution of enzymes and catabolic pathways for the degradation of synthetic compounds has received considerable attention. This has often been based on the notion that the degradation of xenobiotics presents a real challenge for microorganisms in polluted environments. Often, however, the evolution of catabolic pathways for xenobiotics is discussed in the context of systems that have only distant evolutionary relationships. It seems unlikely that such distant similarities are related to, or reflect evolutionary processes that occurred over the last 100 years, as might occur in response to the release of synthetic chemicals. If we want to understand how microorganisms present in the natural environment adapt to the introduction of industrial pollutants into the environment, it is important to focus on evolutionary events which have occurred recently. In fact, very little is known about the genetic changes that have occurred during the exposure and adaptation of microorganisms to industrial chemicals. Even though an organism degrades a chlorinated (xenobiotic) compound, it does not mean that a new catabolic pathway is required, since numerous organohalogenes are naturally produced.

Different views have been presented with respect to the degree of mutations that occur during the evolution of new enzymes. A spectacular hypothesis has been proposed by Ohno (39), who suggests that the nylon oligomer hydrolase gene of *Flavobacterium* sp. K172 had evolved by a shift in reading frame, which occurred in a very ancient gene that was composed of primordial oligomeric repeats and encoded an arginine-rich protein of completely unrelated function. This view supports that a completely new protein arose due to exposure and adaptation to a xenobiotic chemical. However, gene sequences homologous to the oligomer hydrolase can also be found in other bacteria, including organisms with no known history of nylon oligomer degradation, such as *P. aeruginosa* PAO. Furthermore, it is impossible to rule out that a very similar hydrolase exists that is involved in cleavage of amide bonds found in natural compounds. We support the view that new catabolic activities evolve by a limited number of mutations in genes that encode proteins with very similar function.

A natural enzyme for which a recent adaptation process has been proposed is the haloalkane dehalogenase from *X. autotrophicus* GJ10 (45). Several lines of evidence indicate that this dehalogenase is especially adapted to convert haloalkanes, and has undergone the following evolutionary changes during adaptation to synthetic 1,2-dichloroethane:

- The nucleophile consists of aspartate and two tryptophan residues which are involved in stabilization of the leaving group. This indicates that the active site of the enzyme is specifically designed to catalyze dehalogenation reactions. Although the dehalogenase is evolutionarily related to α/β -hydrolase fold enzymes, it most probably was already a dehalogenase before industrial pollution started. Because active dehalogenases have been found in other organisms (e.g., *Mycobacterium*), not selected for growth on chlorinated compounds (21), it is likely that the enzyme preexisted, or was a dehalogenase for some other naturally-occurring compounds prior to being challenged with industrial 1,2-dichloroethane.
- The enzyme actually has evolved from a more primitive dehalogenase by recent genetic adaptation events. Short direct sequence repeats are present in the cap domain, i.e., a 15 bp perfect repeat and a 9 bp imperfect (one substitution) repeat. It is unlikely that these repeats are of ancient evolutionary origin, since it is difficult to envision that the presence of short repeated sequences would be needed for proper enzyme function. Instead, we propose that they are the result of recent mutations (less than 100 years ago), which adapted the enzyme to 1,2-dichloroethane. This could be achieved by starting from a dehalogenase that was active with an unknown natural halogenated compound.
- Experimental work supports the role of spontaneous mutants has been investigated. The mutations selected are all localized in the cap domain, and included repeats similar to of those identified in the wild-type enzyme (45).

This 1,2-dichloroethane dehalogenase is probably the only dehalogenase for which there is some evidence that it has undergone mutations during adaptation to a synthetic organohalogen. Although speculation exists, there is little proof for recent adaptations to halogenated substrates of, for example, glutathione transferases and enoyl hydratases. There certainly is an evolutionary relationship between these latter enzymes and the dehalogenases, but the time of branching may have occurred very long ago, and may not be related to adaptation to the relatively recent presence of xenobiotics. Again, it should be noted that many natural organohalogens exist, and that haloaromatics are particularly abundant, e.g., in decomposing wood (see also Chapter 1 and 6).

Features in the DNA sequence which are the result of fortuitous mutations and serve no useful function should be regarded as a sign of recent genetic change, as opposed to those evolutionary changes that are the result of continued selection and optimization of enzyme activity (42). A striking example of how the sequence of a gene that has undergone recent mutations may be influenced by the mutagenic event is provided by the haloalkane dehalogenase gene of *Mycobacterium* sp. GP1 (Figure 7.3). The *dhaA_r* gene of this organism is fused in frame to a segment of the *hheB* gene, encoding haloalcohol dehalogenase, resulting in an extension of the *dhaA* open reading frame by 14 codons. Since this *hheB* part of *dhaA_r* is identical to the 3' end of an intact *hheB* gene that is located approximately 2.6 kb further downstream, it seems likely that a duplication of the *hheB* gene occurred prior to acquisition and insertion of the *dhaA* gene. The organization and sequence of these catabolic genes as we see it now, is in part the result of fortuitous genetic events that yielded a suitable activity which probably holds for other systems as well. Moreover, it does not appear to be the result of extensive evolutionary optimization.

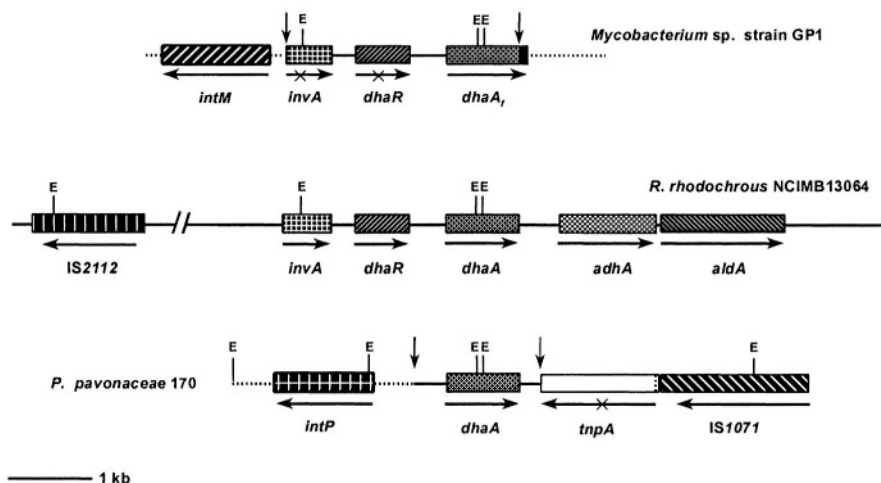


Figure 7.3. Comparison of the genetic organization of the haloalkane dehalogenase gene region in *Rhodococcus erythropolis* NCIMB13064, *Pseudomonas pavonaceae* 170, and *Mycobacterium* sp. GP1. It is proposed that *Rhodococcus* DNA is the genetic origin for the *Mycobacterium* and *Pseudomonas* *dhaA* regions. In *Mycobacterium* sp. GP1, the *dhaA* gene is fused to a segment of a haloalcohol dehalogenase gene, creating *dhaAf*, and a deletion has occurred in the repressor gene *dhaR*, causing constitutive expression. In *P. pavonaceae* 170, the repressor gene is absent, again allowing constitutive expression. The two deletions in the acquired genetic element are indicated by dotted lines. A putative transposase ORF (*tnpA*), which is located next to the acquired genetic element, is interrupted by an insertion element, identified as *IS1071*. The *intP* and *intM* genes encode putative site-specific DNA integrases (40).

5. REGULATION OF DEHALOGENASE ACTIVITY

A dehalogenase enzyme may be acquired by a microorganism and altered to convert a novel compound, but this only contributes to a functional catabolic pathway if the protein is produced when it is needed. Acquisition of a regulatory system for gene expression is a fundamental second step in the evolution of an efficient new catabolic pathway. In addition to the catabolic enzyme, it requires a second protein molecule that can recognize and bind the target compound. The expression of various dehalogenases is indeed regulated by the presence of substrate, which has been identified for enzymes converting halocarboxylic acids, chloroalkanes, chloroacrylic acids, chloroalcohols, 4-chlorobenzoate, and pentachlorophenol. Such inducible expression suggests that the catabolic pathways are well developed. Apparently, there has been enough time to evolve a regulatory system.

A number of regulatory systems have been characterized in more detail. In *Pseudomonas putida* PP3, the regulatory protein DehR1 responds to inducers such as 2-monochloropropionate and monochloroacetate, and activates transcription of the

haloalkanoic acid dehalogenase gene *dehI* (56-58). Transcription is mediated by an alternative RNA polymerase containing the σ^{54} factor. In dichloromethane-utilizing methylotrophs, the negative regulator DcmR controls expression of dichloromethane dehalogenase DcmA (27, 28, 48). The plasmid-located haloalkane dehalogenase gene (*dhaA*) in various haloalkane-utilizing strains of *Rhodococcus erythropolis* is regulated by the product of the adjacent *dhaR* gene (41). The DhaR protein belongs to the TetR family of transcriptional repressor-type regulators and responds to 1-chlorobutane and several other 1-halo-*n*-alkanes (5, 40). It is likely that the regulatory proteins for these enzymes were designed originally to respond to natural organohalogenes, and that they have been modified during the development of degradative pathways for the respective halogenated xenobiotics.

In other cases, a regulatory system is lacking or it is not functional, which may be seen as a sign of evolutionary primitivity. For example, in *X. autotrophicus* GJ10 (Figure 7.1), the haloalkane dehalogenase gene (*dhlA*) is expressed constitutively and addition of 1,2-dichloroethane does not enhance expression. However, an open reading frame that is located directly upstream of the dehalogenase gene encodes a putative regulatory protein that shares low but significant sequence similarity with members of the TetR family of repressor-type regulators (20). In addition, the promoter region of the haloalkane dehalogenase gene contains two copies of the palindromic sequence TAGGTCNNNGACCTA, which may serve as binding sites for the putative repressor. These observations suggest that normal transcriptional regulation of the *dhlA* gene (or its ancestral form) has been relaxed to allow expression in the presence of 1,2-dichloroethane, which is not recognized by the regulator.

Another example in which alteration of a regulatory protein played a role in the ability to utilize a new carbon source is found in *Mycobacterium* sp. strain GP1 (40, 42). This bacterium is capable of growth on 1,2-dibromoethane (Figure 7.1) via a haloalkane dehalogenase (DhaAf), for which 1,2-dibromoethane is a substrate but not an inducer. A deletion of 12 nucleotides has occurred in the regulatory *dhaR* gene, which in other strains encodes a repressor. Although this deletion is in frame, it inactivates the DhaR protein, leading to constitutive expression of the dehalogenase.

6. MOBILIZATION AND DISTRIBUTION OF DEHALOGENASE GENES

6.1. Plasmids, Transposons and Insertion Elements

Various studies on the evolution of catabolic pathways for haloaliphatics have provided evidence that gene transfer plays an important role. Important aspects are:

- Dehalogenating systems are often encoded on self-transmissible plasmids. This has been found for several haloalkane dehalogenases, such as the *dhlA* genes of *Xanthobacter* and *Ancylobacter* strains, the *dhaA* genes of *Rhodococcus* strains, and the *dcm* genes of dichloromethane-degrading bacteria (9, 26, 41, 48, 54).
- Gene clusters encompassing dehalogenases are often associated with insertion elements and located in a transposon-like structure, as has been found in chloropropionate, dichloromethane, and chloroalkane-utilizing organisms. For example, the highly conserved dichloromethane degradative gene (*dcm*) region in several phylogenetically distinct methylotrophic bacteria is associated with three

distinct insertion elements (48). Another example is insertion element *IS1071*, first detected in a gene region involved in chlorobenzoate metabolism. The chlorobenzoate catabolic genes (*cbaABC*) of *Alcaligenes* sp. strain BR60 have been found to be encoded on a composite transposon, designated Tn5271, that is flanked by two copies of this 3.2 kb insertion element (*IS1071*) (64). Subsequently, the same insertion element was shown to flank the haloacetate dehalogenase gene *dehH2* on plasmid pUOI in *Moraxella* sp. strain B (23, 64), the haloalkane dehalogenase gene *dhaA* on the chromosome in *Pseudomonas pavonaceae* 170 (40), the aniline degradative genes on a plasmid of *Pseudomonas putida* UCC22 (8), and presumably also the *p*-sulfobenzoate degradative genes (*psbAC*) on plasmids of *Comamonas testosteroni* (22). *IS1071* thus seems to be responsible for mobilizing catabolic genes to the chromosome or a plasmid in different host organisms.

How an insertion element may transpose itself to a dehalogenase gene and mobilize this gene into a plasmid has been demonstrated by studies on the adaptation of *X. autotrophicus* GJ10 to toxic concentrations of monobromoacetate (59). Bromoacetate can be hydrolyzed by haloacid dehalogenase (*DhlB*), but substrate concentrations above 5 mM are toxic to strain GJ10. Mutants which are able to grow in the presence of higher concentrations of bromoacetate were found to overexpress haloacid dehalogenase, and it appears that this resistance is accompanied by the incorporation of an unlinked insertion element (*IS1247*) to an upstream site adjacent to the chromosomally located *dhlB* gene. This insertion causes increased expression of haloacid dehalogenase. Furthermore, one-ended transposition of *IS1247*, together with *dhlB*, can lead to insertion of a copy of the *dhlB* gene into a plasmid present in the host. A dehalogenase gene thus may become activated by an insertion element, and the combination of a single insertion element and dehalogenase gene can transpose to another replicon.

Similar evolutionary changes have been observed earlier by Senior and coworkers (49). Their continuous culture selection experiments have revealed that during a period of thousands of hours, one member of a seven-membered microbial community capable of utilizing the herbicide 2,2-dichloropropionic acid (Dalapon) acquired the ability to grow on Dalapon through the production of two dehalogenases. Apparently, cryptic dehalogenase genes in the original strain became activated during prolonged selection, most likely through the action of insertion elements (50). Several years later, Thomas *et al.* (56, 57) showed that the dehalogenase gene *dehI* of *P. putida* PP3 indeed is located on a transposable element, designated DEH.

6.2. Do DNA Integrases Play a Role in the Acquisition of Dehalogenase Genes?

Acquisition of foreign DNA by horizontal gene transfer requires integration into a replicon that is stably maintained in the recipient microorganism. Both in *P. pavonaceae* 170 and in *Mycobacterium* sp. strain GP1 a gene encoding a putative site-specific recombinase (*intP* in strain 170 and *intM* in strain GP1), is present directly upstream of the recruited DNA segment harboring the haloalkane dehalogenase gene (Figure 7.3) (40). The *intP* and *intM* gene products share significant sequence similarity with members of the integrase (Int) family of site-specific recombinases, and both harbor the conserved, catalytically important, tetrad R-H-R-Y of the Int family. These putative

integrases probably mediate the insertion of the dehalogenase genes into the genome, although their activity remains to be established experimentally. The finding of putative integrase genes next to the dehalogenase genes further reinforces our hypothesis that these DNA segments were acquired by horizontal transmission.

Integrase-mediated gene acquisition has previously been associated with a class of genetic elements called integrons (51). Integrons harbor a gene for a site-specific DNA integrase, which can mediate the incorporation of one or more foreign genes in a specific site (the recombination or core site), directly upstream of the integrase gene. Thus far, integrons have been implicated in the acquisition of antibiotic resistance genes by various bacterial species.

6.3. Global Distribution of a Gene Cluster Encoding Haloalkane Catabolism

In some cases, organisms possessing identical dehalogenase genes have been found in very different geographical areas. For example, Gram-negative methylotrophs containing identical or nearly identical *dhlA* or *dcmA* genes have been isolated from widely separated sites in Europe. Furthermore, we have found that haloalkane-utilizing *Rhodococcus* strains isolated from contaminated sites in Europe, Japan, and the United States possess identical haloalkane dehalogenase (*dhaA*) genes (41). The same gene is also present in the 1,3-dichloropropylene degrader *P. pavonaceae* 170 and, in slightly modified form, in the 1,2-dibromoethane degrader *Mycobacterium* sp. GP1. Thus, transfer of the *dhaA* gene from a *Rhodococcus* strain to a Gram-negative organism may have occurred. Apparently, the capacity to use haloalkanes as a carbon source has become widespread due to the recent and global distribution of a single catabolic gene cluster. In all strains analyzed, the gene cluster is localized on plasmids or associated with insertion elements, suggesting a role for these mobile elements in gene transfer.

Such observations are difficult to interpret without considering long-distance distribution mechanisms for microorganisms. Despite growing concern about biological invasions and emergent diseases, not much is known about the long-distance distribution mechanisms for microorganisms. Recently, Ruiz *et al.* (47) have suggested that the global movement of ballast water in ships creates a long-distance dispersal mechanism for human pathogens. Similarly, the global movement of seed and agriculture food products (e.g., potatoes, fruits and vegetables) may have contributed to the global spread of organohalogen-degrading microorganisms. Agricultural products are often treated with halogenated fumigants and/or are harvested from soils treated with fumigants, such as the soil disinfectants 1,3-dichloropropene and methyl bromide (see Chapter 12). This may have created ecological niches where dehalogenase genes proliferate, and thereby increased their occurrence and distribution.

7. WHY ARE SOME COMPOUNDS STILL RECALCITRANT?

Several important chlorinated aliphatics that occur as environmental pollutants have so far not been found not to serve as a growth substrate for bacterial cultures under aerobic conditions. This includes compounds such as 1,1-dichloroethene, 1,2-dichloropropane, 1,2,3-trichloropropane, the 1,2-dichloroethenes, trichloroethanes, and

trichloroethene. For all these compounds, some form of degradation under aerobic conditions has been demonstrated. Thus, 1,1-dichloroethane may be slowly oxidized by monooxygenases, 1,2-dichloropropane and 1,2,3-trichloropropane are (poor) dehalogenase substrates, and the dichloroethenes can be converted to their epoxides by monooxygenase activity. Rates of transformation are very low, however, or the products of the initial conversions are toxic, as with the chloroethene epoxides. There are no fundamental reasons that prohibit the use of these compounds as a substrate for aerobic growth. Their current recalcitrance may well be a fortuitous situation, and with ongoing evolution and adaptation, new organisms may appear with time. Of course, the time needed to evolve new metabolic activities is of great practical importance. Since evolution processes can be accelerated under laboratory conditions, it is entirely possible that directed evolution techniques will contribute to obtaining organisms with enhanced catabolic potential.

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